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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4-6
Key Research Accomplishments.....	6-8
Reportable Outcomes.....	8
Conclusions.....	8-9
References.....	9
Appendices.....	10-17

Introduction

In the field of breast cancer, the significant cytoreduction achieved by high-dose chemotherapy with autologous progenitor cell support or recombinant hematopoietic growth factors remains an important strategy. The role of consolidation after high dose chemotherapy has been accepted to reduce disease relapse in breast cancer. However, toxicities of the chemotherapeutic agents, in particular myelosuppression, still impose restrictions on the optimization of this treatment modality. Insertion of drug resistance genes into hematopoietic progenitor cells offers an additional approach to allow further dose intensification and treatment post transplant. We have generated and characterized human thymidylate synthase (TS) mutants, one of which resulted in a 15-fold increase K_i for 5-fluoro-2-deoxyuridylate (FdUMP) compared to the wild type TS (Tong et al). This mutant TSG52S (glycine at position 52 was replaced with serine) transfectant confers a 97-fold resistance to fluorodeoxyuridine (FdUrd) in TS negative mouse cell lines. 5-fluorouracil (5-FU), is a commonly used drug in breast cancer treatment with significant myelosuppressive side effects. We hypothesize that after administration of high dose chemotherapy, transplanted progenitor cells transduced with the fluoropyrimidine drug resistance gene, TSG52S, will allow for higher doses of 5-FU to be given during maintenance therapy without significant myelosuppression possibly leading to improved cure rates. Therefore, the overall objective is to develop retroviral vectors and efficient viral transduction methods for introducing TSG52S into mouse and human hematopoietic progenitor cells and then to evaluate the level of resistance to myelosuppression from 5-FU this method produces.

Body

Specific Aims

We plan to:

- 1) to construct retroviral vectors which contain the mutated TS (G52S) with and without mutated dihydrofolate reductase (DHFR) and mutated TS (G52S) with enhanced green fluorescent protein (EGFP). DHFR and EGFP are selectable markers which improve detection of transduction.
- 2) use these constructs to determine the efficiency of gene transfer and their ability to confer 5-FU resistance in vitro, using 3T3 cells and an HL60 cells as test systems. Mouse bone marrow cells will be used for in vitro CFU-GM assay.
- 3) test the hypothesis that gene transfer of the mutated TS (G52S) into hematopoietic cells protects them from high dose chemotherapy related myelosuppression in a mouse model.
- 4) test and optimize conditions for transduction of CD34 separated human hemtopoietic progenitor cells (peripheral blood stem cells) using these constructs.

During the first year, we have accomplished,

- 1) Retroviral vectors containing a mutated TS and a mutated DHFR or these with EGFP marker both were able to infect NIH3T3 cells and conferred resistance to 5-FU and MTX.
- 2) The human CD34+ enriched cells infected with these vectors also conferred resistance to 5-FU, MTX, or both 5-FU/MTX combination treatment.

Key Research Accomplishments for this year

We have completed the aim 3) during this year award period.

- 3) test the hypothesis that gene transfer of the mutated TS (G52S) into hematopoietic cells protects them from high dose chemotherapy related myelosuppression in a mouse model.

Methods:

Infection of mouse bone marrow cells, bone marrow transplantation, and treatment of recipients

Bone marrow cells from BD2F1 (7-11 week old male) donor mice were treated with 5-FU (150 mg/kg). After 4 days, bone marrow cells from the mice were harvested and resuspended in IMDM medium, and a mononuclear cell suspension was prepared. The recovered mononuclear cells were then co-cultured with the amphotropic virus-producing cell line that had been irradiated with 1500cGy 2 hours previously. The co-culture was carried out in the condition described previously (Takebe *et al.*, 2001). 2×10^6 non-adherent cells were injected via the tail vein into lethally irradiated mice (900cGy whole body, 24 hours previously). Control mice received bone marrow cells treated in the identical fashion, but incubated with the non-virus producing AM12 packaging cell line. The drug administration schedule for the experiment was as follows: the mock-transduced group, the SFG-F/S-TS52 (fusion) vector-transduced group, the SFG-EGFP-IRES-F/STS52 (EGFP-fusion) vector-transduced group, and the SFG-TS52-IRES-F/S vector-transduced group, each group containing six animals (total of 36 animals), were treated with 5-FU 75 mg/kg/day for 3 days, day 26, 27, and 28, and MTX 600 mg/kg x 1 on day 26.

Mouse bone marrow CFU-GM assays

After completion of retroviral infection by the coculture method as described above, an aliquot of bone marrow cells used for the mouse in vivo bone marrow transplantation was submitted for CFU assays. In addition, bone marrow cells from the mouse recipient receiving bone marrow transplantation and high dose 5-FU and MTX were harvested and analyzed for CFU assay. The bone marrow cells were added in 10 x 35-mm plates to 2 ml of semisolid medium containing 1% methylcellulose, thymidine phosphorylase-treated 20% FBS. 10% WEHI-3B conditioning medium, 1% NaHCO₃, 1% sodium pyruvate, 1μM β-mercaptoethanol, 100U/ml penicillin, 100μg/ml streptomycin, 1% essential amino acids, 1.5% nonessential amino acids, and 0.5% vitamin C. The cells were incubated with increasing doses of MTX, 5-FU, or both. The plates were kept at 37°C in a 5% CO₂ incubator, and colonies of >50 cells were scored after 12 days.

Measurement of hematologic parameters of mice and demonstration of proviral DNA in mouse bone marrow cells by PCR

White blood cells (WBC), platelets counts, weights, and survival were monitored in all groups of animals. Proviral DNA detection of the human TS cDNA and EGFP cDNA in bone marrow cells from animals receiving SFG-EGFP-IRES-F/S-TS52 (EGFP-fusion) transduced bone marrow cells was evaluated by PCR method. The detection of TS was demonstrated as a result of amplification of a 485 bp fragment from genomic DNA using the TS2A and TS6A primers described as above. EGFP cDNA was detected by amplification of a 300 bp fragment using the EGFP1 and EGFP2 primers. PCR analysis was carried out for 40 cycles in 50μl reaction mixtures containing genomic DNA

prepared from mouse tissues. 1.25 mmol/L of each dNTP, 1 x PCR buffer (Perkin Elmer Cetus), 1 µl of each primer (300ng/µl) and 0.5µl of Taq polymerase (Perkin Elmer Cetus). PCR conditions for TS amplification were as follows; 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes for 40 cycles, EGFP amplification; 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 1 minute for 40 cycles. The PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. EGFP primer sequences are described as follows.

EGFP1: 5'-GCCACAAGTTCAGCGTGTCC-3'

EGFP2: 5'-AGCTCGATGCGGTTTACCAG-3'

The colonies were also examined under fluorescence microscope for EGFP expression.

Results:

Mouse bone marrow transplantation (BMT) and drug treatment.

The experiment was performed in mice transplanted with three different vector transduced bone marrow cells, SFG-TS52-IRES-F/S-, SFG-F/S-TS52-, and SFG-EGFP-IRES-F/S-TS52 (Appendix 1a, 1b, 1c, 1d). 5-FU treatment was given on days 26, 27, and 28 post transplant at a dose of 75 mg /kg /day. MTX treatment was given on day 26 at 600mg/kg x 1 post transplant. Five out of 6 animals in both SFG-F/S-TS52- and SFG-EGFP-IRES-F/S-TS52-transduced groups survived through the experiment (followed until day 60), 4 out of 6 animals in SFG-TS52-IRES-F/S transduced group survived, whereas 6 out of 6 animals in mock-transduced group died from chemotherapy toxicity (Figure 6a).

The animal weight changes during the experiment showed both SFG-EGFP-IRES-F/S-TS52 and SFG-F/S-TS52 groups continued to lose weight until post BMT day 45.

However, the weight returned back to their baseline around post BMT day 60 (Appendix 1b). The SFG-TS52-IRES-F/S group continued to lose weight until post BMT day 50 and finally the weight started to recover close to their day 0 weight level. The mock group animals all developed significant weight loss and died by post BMT day 50. The animal WBC count in both SFG-EGFP-IRES-F/S-TS52 and SFG-F/S-TS52 groups showed the nadir around post BMT day 35 to 45. However, the nadir did not dip more than $10 \times 10^3 / \mu\text{l}$. The SFG-TS52-IRES-F/S group developed nadir around post BMT day 40 when their WBC count was around $5-6 \times 10^3 / \mu\text{l}$. The mock group animal developed significant neutropenia around post BMT day 50 and all died from toxicity (Appendix 1c). The platelet count recovery was also tracked and both SFG-EGFP-IRES-F/S-TS52 and SFG-F/S-TS52 group showed the maximum thrombocytopenia developed around post BMT day 45. The pattern of thrombocytopenia in SFG-TS52-IRES-F/S group followed the pattern of both fusion construct groups. However, the mock group animals developed significant thrombocytopenia and died from toxicity (Appendix 1d).

Mouse bone marrow CFU-GM assay

We performed CFU-GM assay using mouse bone marrow cells transduced with three different vectors as described in the "mouse bone marrow transplantation (BMT) and drug treatment" section. In order to compare the result of drug resistance in CFU-GM assay between bone marrow cells collected before BMT and after BMT followed by high

dose chemotherapy treatment in vivo, we performed CFU-GM from bone marrow cells after viral transduction and from bone marrow cells post BMT day 60 (Table 1a and 1b). Colony count from the non-drug treatment group was determined as 100% and each colony count with different drug concentration exposures was calculated as percent survival. Each condition was repeated in triplicate and the data shows the summation of colony counts from the three plates along with the calculated percentages. CFU-GM assay using bone marrow cells immediately after vector transduction (Table 1a) showed increased resistance to 5-FU at 10^{-6} M in all three vectors, SFG-TS52-IRES-F/S (20%), SFG-F/S-TS52 (26%), and SFG-EGFP-IRES-F/S-TS52 (25%), whereas mock transduced group showed 4% survival. MTX exposure at 2×10^{-8} M showed 13%, 17%, and 18%, and 0% survival for SFG-TS52-IRES-F/S, SFG-F/S-TS52, SFG-EGFP-IRES-F/S-TS52, and mock, respectively. Combined exposure with both 5-FU and MTX together with the same drug concentration showed colony survival was seen in all vector transduced groups. No colony survival was seen in the mock group.

Mouse bone marrow CFU-GM was also performed using bone marrow cells harvested from animals survived through BMT and chemotherapy (Table 1b). Control bone marrow cells were obtained from untreated mice since control animals received BMT and chemotherapy were 100% lethal. The result from this experiment showed similar to pre-BMT CFU-GM result as described above. This result confirmed that post BMT day 60 bone marrow cells were able to confer resistance to both 5-FU and MTX.

We also analyzed EGFP expression conferred by SFG-EGFP-IRES-F/S-TS52 construct by fluorescence microscopy. Mouse bone marrow cells were harvested after BMT followed by chemotherapy and submitted for CFU-GM. CFU-GM without drug treatment showed 19% green fluorescent colonies (10 / 52 colonies) which was consistent with MTX / 5-FU treated CFU-GM colony survival percentage (21%).

Detection of proviral DNA in mouse bone marrow cells 60 days after transplant

We analyzed the detection of proviral DNA from mouse bone marrow cells which were harvested from mice received SFG-EGFP-IRES-F/S-TS52 transduced bone marrow cell transplantation followed by high dose 5-FU and MTX challenge and kept until day 60 post BMT. One of the mice surviving this procedure was sacrificed and bone marrow cells were used for CFU-GM assay as described above. Colonies were harvested to obtain DNA which was used as template for PCR amplification using either human TS or EGFP primers to amplify TS52 cDNA fragment or EGFP cDNA fragment (see Methods). PCR primers used in this experiment (forward; TS2A and reverse; TS6A as described in Methods) were able to amplify TS cDNA with 485 bp fragment size in 3 out of 7 colonies (43%) (Figure 2a). We also amplified EGFP cDNA fragment of 300bp size using EGFP primers described as above (see Methods). Three out of 8 colonies (38%) were detected as positive by showing the expected size fragments (Figure 2b). Mock transduced cells as well as negative control for PCR analysis were negative by using either of primers.

We analyzed proviral DNA in bone marrow cells before submitting to CFU-GM from same animals. We observed expected size of PCR amplified EGFP cDNA bands and TS52 cDNA band in DNA gel electrophoresis (Figure 3).

Table 1a

Pre-BMT
5-FU and MTX resistance in virally transduced mouse bone marrow cells CFU
Number of colonies (%)

	No Drugs	5FU [10 ⁻⁶ M]	MTX [2 x 10 ⁻⁸ M]	5FU+ MTX
AM12	302(100)	12 (4)	0 (0)	0 (0)
TS52-I-F/S ^a	314(100)	63 (20)	41 (13)	44 (14)
F/S-TS52	296(100)	71 (26)	50 (17)	56 (19)
EGFP-I-F/S-TS52 ^b	290(100)	73 (25)	52 (18)	52 (18)

Note: Mouse bone marrow cells were infected by coculture with amphotropic viral producer cells (see Methods). CFU-GM colonies obtained with and without 5-FU or MTX are indicated.

^a TS52-I-F/S represents the TS52-IRES-F/S construct, ^b EGFP-I-F/S-TS52 represents the EGFP-IRES-F/S-TS52 construct-transduced mouse bone marrow cells.

Table 1b

Post-BMT
5-FU and MTX resistance in virally transduced mouse bone marrow cells CFU
Number of colonies (%)

	No Drugs	5FU [10 ⁻⁶ M]	MTX [2 x 10 ⁻⁸ M]	5FU+ MTX
AM12	288(100)	14 (5)	0 (0)	0 (0)
TS52-I-F/S ^a	296(100)	53 (18)	44 (15)	47 (16)
F/S-TS52	268(100)	64 (24)	51 (19)	59 (22)
EGFP-I-F/S-TS52 ^b	266(100)	61 (23)	53 (20)	56 (21)

Note: Mouse bone marrow cells infected by coculture with amphotropic viral producer cells were transplanted into mice followed by high dose chemotherapy. The surviving mice were sacrificed and bone marrow cells were harvested on day 60 post BMT. The cells were analyzed for colony assays (see Methods). CFU-GM colonies obtained with and without 5-FU or MTX are indicated.

^a TS52-I-F/S represents the TS52-IRES-F/S construct, ^b EGFP-I-F/S-TS52 represents the EGFP-IRES-F/S-TS52 construct-transduced mouse bone marrow cells.

Reportable Outcomes

None

Conclusions:

We used three different vector constructs in *in vivo* mouse BMT experiment. The mice recipients which received SFG-TS52-IRES-F/S-transduced bone marrow cells were used as the non-fusion vector control. Both the fusion vectors SFG-F/S-TS52 and SFG-EGFP-IRES-F/S-TS52 showed comparable protection of bone marrow protection to non-fusion

vector, by demonstrating 5 out of 6 mice survival in both fusion vector groups compared to 4 out of 6 mice survival in the non-fusion vector group. This result is considered to be the result of ameliorated leukopenia after high dose chemotherapy challenge. The non-fusion group developed the maximum WBC count reduction of 75% on day 40 post BMT followed by a rapid WBC recovery compared to approximately 50% reduction of WBC count followed by a rapid leukopenia recovery in the fusion vector groups. The mock transduced group continued to drop WBC count even after day 40 post BMT and never showed any WBC recovery. The fusion vector groups showed the maximum platelet reduction of 50% which was comparable to the non-fusion vector group. The mock group continued to have decreased platelet count and never recovered. Animal weight changes in the fusion vector groups showed the maximum weight drop occurred on day 45 followed by weight recovery. However, the non-fusion vector group showed the maximum drop on day 50. This weight change pattern was different from the fusion vector groups and it may be considered as an improved toxicity reduction conferred by fusion vectors compared to that of non-fusion vector.

We analyzed mouse bone marrow CFU-GM assay pre- and post-BMT to see any changes in percentage of progenitor cells conferring drug resistance *in vitro*. Post BMT marrow cells after high dose chemotherapy challenge *in vivo* (day 60 post BMT) were harvested. The percentage of drug resistant CFU-GM colony did not show any significant difference from pre-chemotherapy challenged marrow cells. We did not perform the parallel study comparing animals without drug challenge *in vivo* to compare CFU-GM assay with the animals which were not treated with chemotherapy but received vector transduced cells. Because of this reason, we cannot conclude that one time dose of chemotherapy administration did not have any effect on transduced cell selection or enrichment. To further explore this subject, we need to perform serial high dose chemotherapy administration or serial BMT along with drug treatment to follow EGFP percentage changes *in vivo* and CFU-GM.

CFU-GM colonies obtained after BMT and high dose chemotherapy were tested for DNA PCR for the detection of human TS and EGFP. The detection rate for TS and EGFP was 43% and 38%, respectively, which was greater than the CFU-GM colony survival rate of approximately 20-23% with drug exposures. EGFP DNA detection rate was also greater than the rate of positive fluorescence (19%) by fluorescence microscopy analysis. This may be possible due to the vector silencing or insufficient gene expression. From this experiment, we found that vector transduction efficiency and vector gene expression efficiency may be different especially in the setting of *in vivo* animal studies.

References:

Takebe N, Zhao SC, Adhikari D, Mineishi S, Sadelain M, Hilton J, Colvin M, Banerjee D, Bertino JR. generation of dual resistance to 4-hydroperoxy-cyclophosphamide and methotrexate by retroviral transfer of the human aldehyde dehydrogenase class 1 gene and a mutated dihydrofolate reductase gene. 2001 Molecular Ther 3, 89-96.

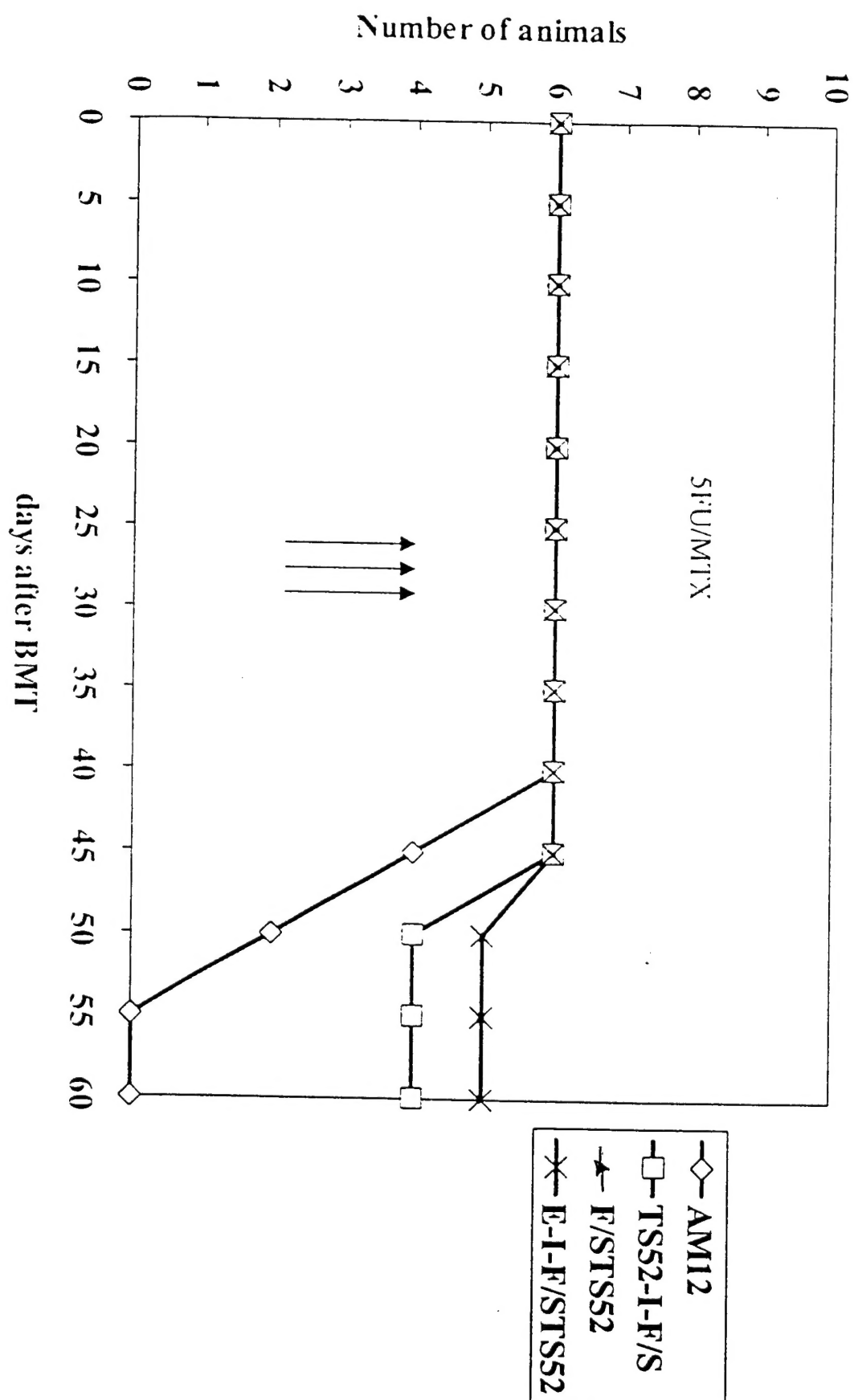
Appendices:

Appendix 1a-1d

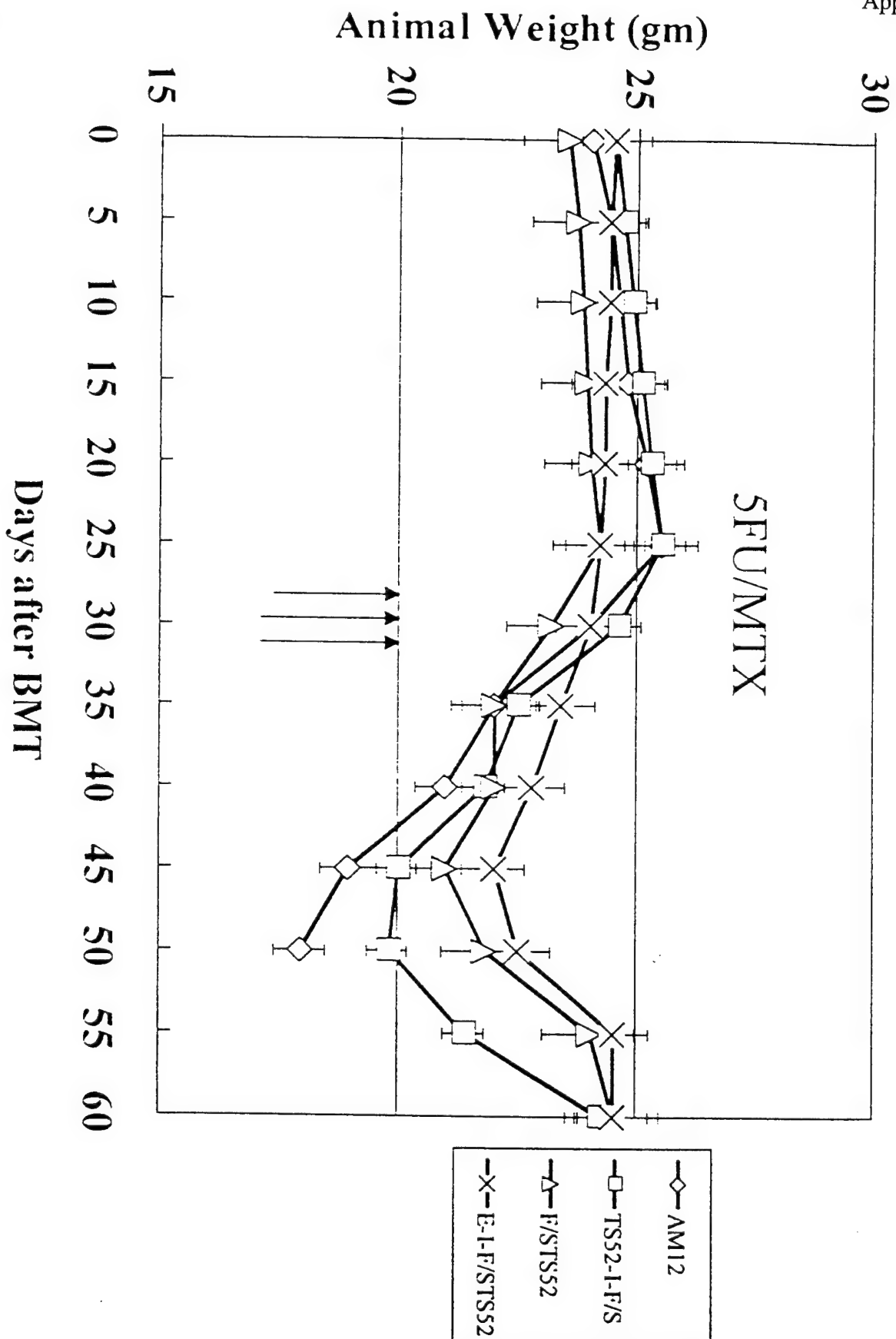
Appendix 2

Appendix 3

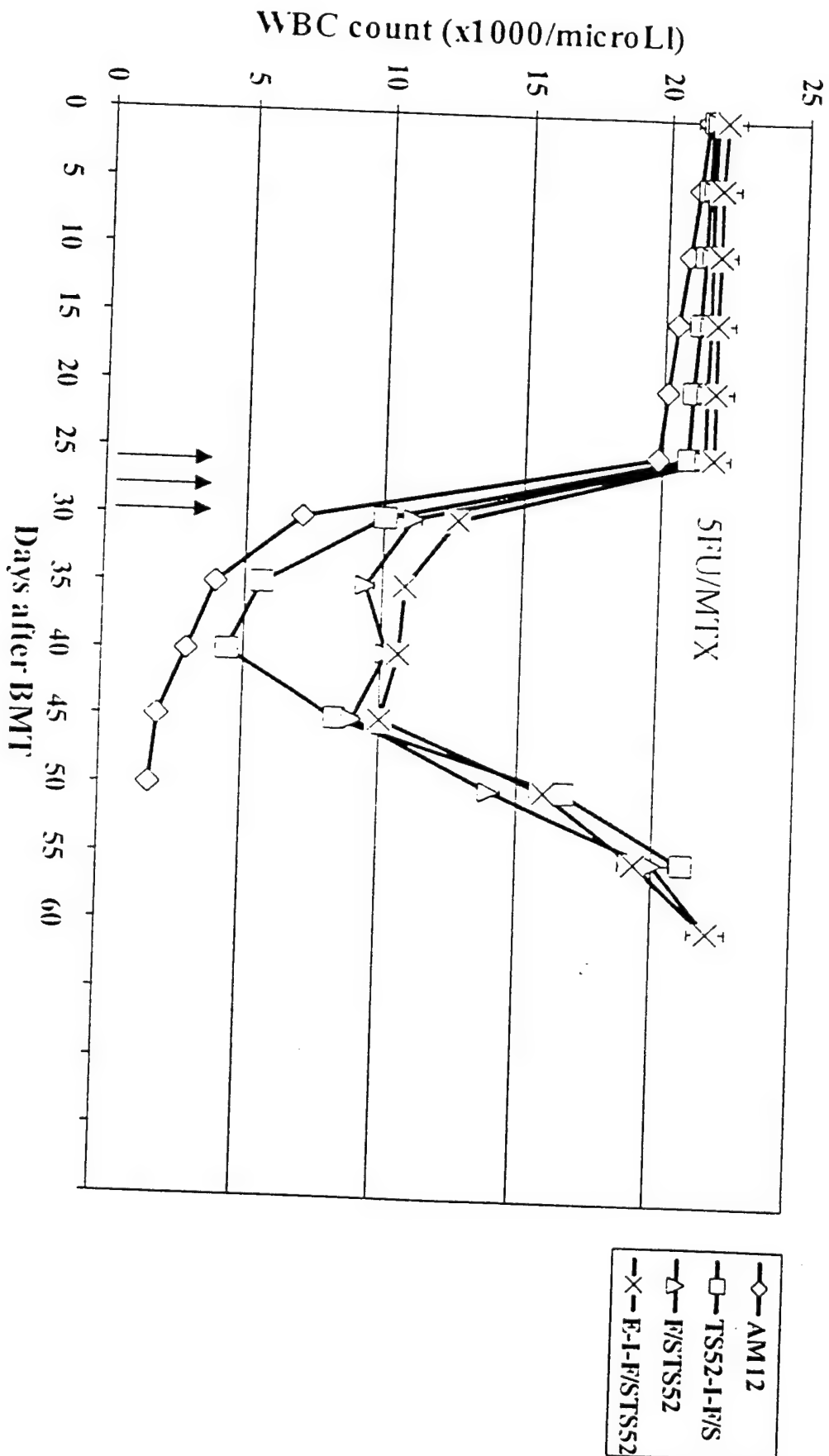
Number of surviving animals after BMT and 5FU/MTX



Animal Weight after BMT and 5FU/MTX

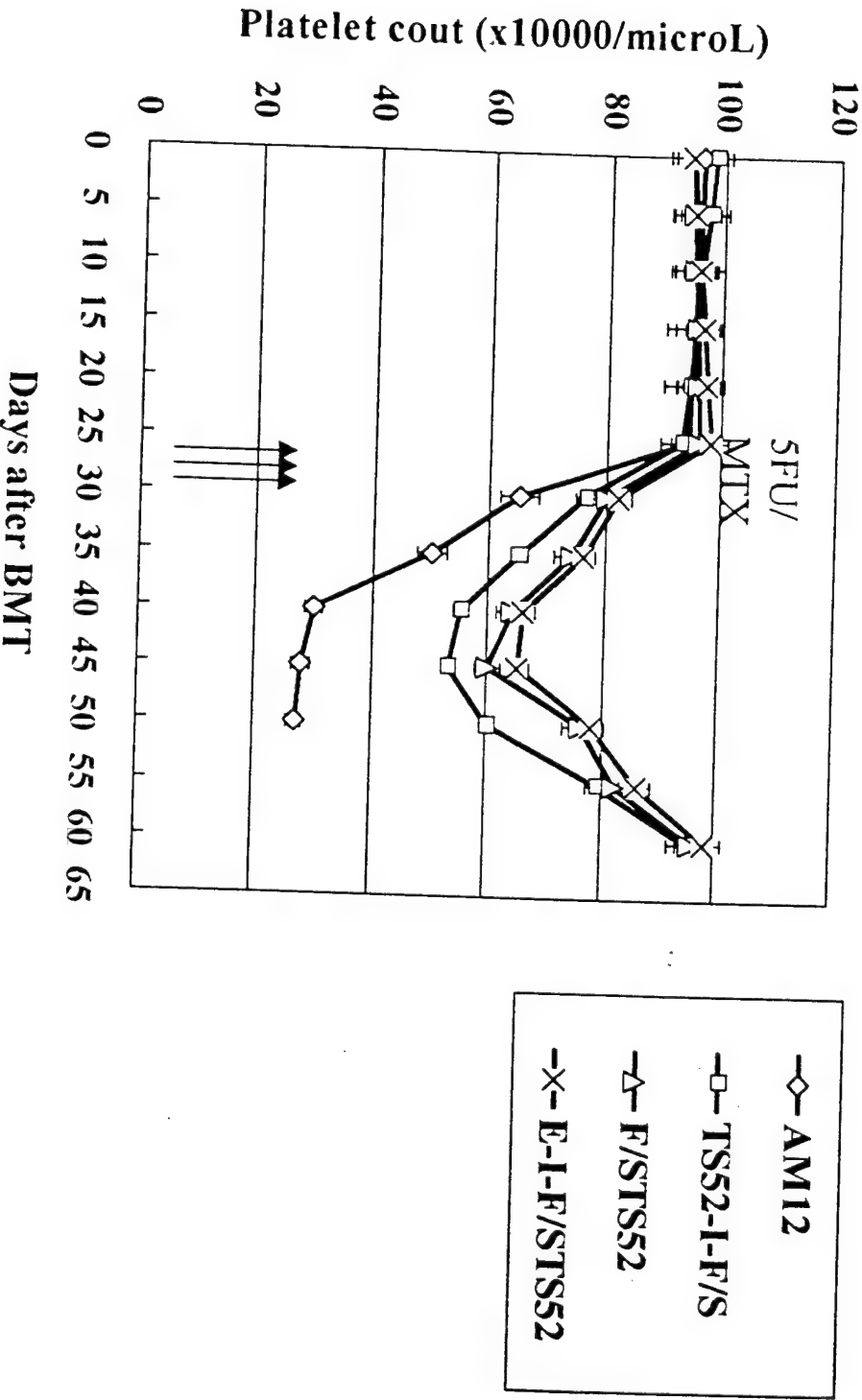


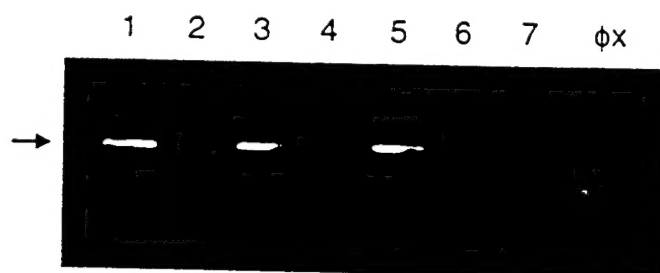
WBC count after BMT and 5FU/MTX

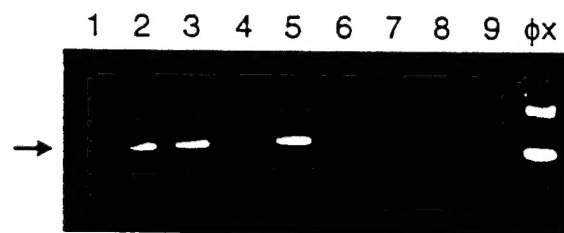


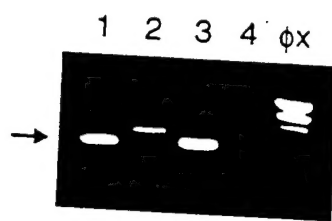
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Platelet count after BMT and 5FU/MTX









Legend: Appendix 1a-d.

Appendix 1. Mouse bone marrow transplantation and drug treatment.

Six animals from each group were transplanted with mouse bone marrow cells transduced with SFG-TS52-IRES-F/S (—□—), SFG-F/S-TS52 (—▲—), SFG-EGFP-IRES-F/S-TS52 (—X—), or mock (—◇—). (a) The survival of mice after BMT followed by 5-FU (75mg/kg x 3 days) administered on days 26, 27, 28, and MTX (600mg/kg x 1) on day 26. All mice receiving mock-transduced marrow died of toxicity (6 out of 6), whereas 5 out of 6 SFG-F/S-TS52 transduced and SFG-EGFP-IRES-F/S-TS52 transduced group mice survived. Four out of 6 SFG-TS52-IRES-F/S transduced group mice survived. (b) The animal weight. (c) The effect of drug treatment on the WBC count. (d) The effect of drug treatment on the platelet count from the same animals. All the values shown are the means \pm 2SD.

Appendix 2. (a) Detection of proviral DNA in mouse bone marrow cells by PCR amplification. Genomic DNA extracted from CFU-GM colonies originated from mouse bone marrow harvested on day 60 post BMT was prepared (see Methods). (a) Detection of human TS sequences utilized primers as described under methods. Lanes 1-7 were from PCR reactions using DNA from SFG-EGFP-IRES-F/S-TS52 transduced CFU-GM colonies. Samples were run with a molecular weight marker, Φ X174, as shown. Lanes 1,3, and 5 showed expected-size human TS cDNA products (Three positives out of 7 colonies). (b) Same DNA used for TS PCR amplifications was used for PCR reactions using EGFP sequence primers. Lanes 1-8 were from PCR reactions amplified with EGFP primers. Lane 2,3,5 showed expected-size EGFP cDNA products, whereas no products were seen from mock-transduced CFU-GM (Eight negatives were not shown in this picture, instead the PCR from only 1 colony was shown as lane 9).

Appendix 3. Detection of the human TS cDNA and EGFP by PCR amplification from bone marrow of day 60 post BMT recipients.

DNA from 3 surviving animal bone marrow was examined for presence of human TS cDNA by PCR using primers (Lanes 1-3). Lane 4, negative control using mock-transduced bone marrow cells as template DNA. DNA size markers (Φ X174 HaeIII digest) are in the far right lane.